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Sex Identification and the Sex-Related Genes of *Nipponia Nippon*

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Abstract: Sex identification in birds is necessary for studying their ecology, behavior and population dynamics and structure. This study was carried out to determine the sex of *Nipponia nippon* by a Polymerase Chain Reaction (PCR) method. The sexing universal primers of non-ratite birds were employed to amplify the sexing related genes and identify the sex of each individual. A 657 bp CHD gene fragments on Z chromosome in both sexes and a 463 bp female specific CHD fragments on W chromosome were cloned and sequenced. The sequences of these two sexing related genes fragments were similar with CHD-W of *Phalacrocorax capillatuss* and CHD-Z of *Platalea minor* individually. This study is valuable for managing and conserving endangered birds.

Keywords: *Nipponia nippon*, Sex Identification, Sex-Related Gene, CHD Gene Sequences of *Nipponia nippon*

Introduction

Nipponia nippon, also known as the Asian Crested Ibis, is the only member of the genus *Nipponia* and one of the world's rarest birds (Hirschfeld *et al*., 2013). Historically, this species was widespread in the Russian Far East, China, Korea and Japan (Li and Li, 1998). Habitat loss and persecution in last century brought this endangered species to the brink of extinction (Yang *et al*., 2012). It has disappeared from most of its former range, except in Shaanxi province of China, but it has been reintroduced into Japan in 2008 (Nagata and Yamagish, 2013). *N. nippon* has been listing in Appendix I of the conservation treaty the Convention on International Trade in Endangered Species of Wild Fauna and Flora, also known as the Washington Convention (CITES), which is a multilateral treaty to protect endangered plants and animals (Wang *et al*., 2010).

N. nippon is a nationally protected bird in China. In order to protect the species, extensive captive breeding projects have been developed (Xi *et al*., 2001). Accurate sex determination is the primary step in captive breeding and also valuable for studying *N. nippon's* ecology, behavior, population dynamics and structure (Ramos *et al*., 2009). *N. nippon* is sexually monomorphic, meaning that both the males and females are generally similar in morphology, which makes its sex difficult to determine because traditional methods used in the sex determination of birds are time-consuming, expensive and even invasive and harmful (Morinha *et al*., 2012). Consequently, several molecular methods have been developed for identifying the sex of sexually monomorphic birds (Dubiec and Zagalska-Neubauer 2006).

The ZW sex-determination system is a biological system that determines the development of sexual characteristics in birds. Males are the homogametic sex (ZZ), while females are the heterogametic sex (ZW) (Davis, 2010). Since the application of Chromo-Helicase-DNA binding (CHD) gene for sex determination of birds in 1995, the CHD gene has turned into the most important molecular marker in the sex identification of birds (Liu *et al*., 2006). Primers commonly used for gender identification in birds included ratite bird specific primers viz. W1/K7, W5/W7 and non-ratite CHD gene specific primers viz. 2550F/2718R, 1237L/1272H, P2/P3, P2/P8 (Bhatt *et al*., 2016). A ratite is any of a diverse group of large, flightless birds of the infraclass *Palaeognathae*, which have no keel on their sternum. Without this to anchor their wing muscles, they could not fly even if they were to develop suitable wings. *N. Nippon* does not belong to ratite birds. In this study, A system to sex *N. nippon* was established using primers 2550F and 2718R which are the sexing universal primers of non-ratite birds. In order to compare the CHD genes between *N. nippon* and the orther birds, the CHD genes were also sequenced and analyzed, which provide some molecular level materials and should be valuable for carrying out captive breeding programs in *N. nippon*.

Materials and Methods

Sample Collection and DNA Extraction

Fresh bloods were collected from *N. nippon* at the Louguantai Wild Animal Protection and Breeding Center, Zhouzhi county in China. The control blood samples were come from a male and a female bird, whose IDs were respectively No. 144 and 140. All samples were mixed with ACD (Acid Citrate Dextrose) anticoagulants at the ratio of 1:6 and stored at -20°C before analysis. Genomic DNA of *N. nippon* was extracted from blood samples using standard phenol/chloroform methods (Sambrook and Russell, 2002). The quantity and quality of the extracted DNA was measured in a BECKMAN DU640 spectrophotometer.

PCR Conditions and Analysis

The specific primers of non-ratite birds 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') (Fridolfsson and Ellegren, 1999) were used for PCR amplification of a fragment of CHD gene of *N. nippon*. The primers are located in exon regions and amplify a unique intron whose lengths are different between Z and W alleles. The PCR reaction mixture (25 μL) contained 100 ng of DNA templates, 0.2 μM Primers, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.5 U Taq DNA polymerase. The PCR program was as follows: 94°C for 5 min; 35 cycles of 94°C (1 min), 54°C (1 min) and 72°C (2 min); and 72°C for 5 min. The PCR products were visualized after electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Gene Clone and Analysis

The PCR products were purified by Agarose Gel DNA Purification Kit (V-gene xi'an China) according to manufacturer's recommendation. The CHD gene fragments clone were performed according to standard protocols and instruction, which included linkage of the amplification product with the pMD18-T vector (Takara dalian China), transformation into competent *Escherichia coli* (DH5a), screening of the positive clone and plasmid DNA preparation, etc. The cloned insert was sequenced using the universal primer RV-M and M13-47 by Takara Co. China. The sequences of these two sex-related genes fragments were contrasted with CHD-W of *Phalacrocorax capillatuss* (GenBank accession numbers: AB080661) and CHD-Z of *Platalea minor* (GenBank accession numbers: AY464013) using a BLAST search (www.ncbi.nlm.nih.gov/BLAST/) individually.

Results and Discussion

The PCR products were analyzed by agarose gel electrophoresis and the results shown in Fig. 1. There were significant differences between the females and males. A about 450-bp fragment was absent in the males. According to the difference of non-ratite bird karyotypes, we could conclude that the 450-bp approximately fragment was amplified from the CHD-W gene in females. Such a distinct band pattern between the females and males allows for quick and clear sex identification of *N. nippon*.

The DNA sequences of the amplified fragments from the CHD-W gene in females were obtained by cloning and sequencing of PCR products. Comparison of *N. nippon* partial CHD-W sequences with sequences of *P. capillatuss* was shown in Fig. 2. The fragment size of *N. nippon* was 463 bp, while the size of *P. capillatuss* was 459 bp. At the site of the 316th bases, the CHD-W fragment of *N. nippon* had a "CCCC" sequence, wich was absent in *P. capillatuss's*. In addition, there were 23 individual base pair variations detected between the two bird species. This analysis also revealed that the two sequences were 94.38% homologous.

The CHD-Z gene fragments either from famale bird or from male one were found they contained the same sizes (657 bp) and the same nucleotide sequences. The gene sequences of *N. nippon* to *P. minor's* were compared. The result was shown in Fig. 3. The similarity of the sequences was 96.96%. There was a "T" base between 405th and 408th base sites in *N. nippon's* less than in *P. minor's*. In addition, there were 18 individual base pair differences between the two bird species.

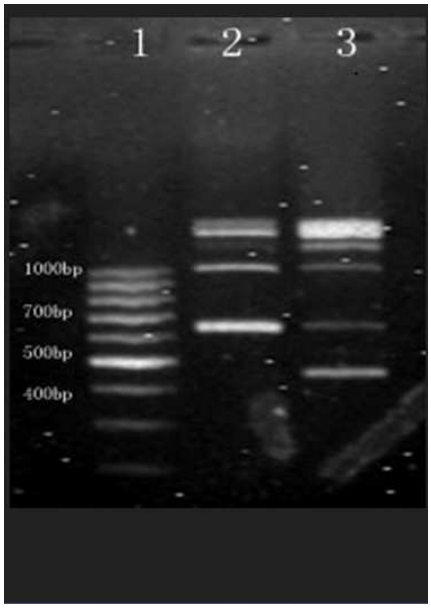

Fig. 1. Sex identification for *N. nippon* using PCR amplification of the CHD genes followed by 1.5% agarose gel electrophoresis. lane 1: Standards, lane 2: Male, lane 3: Female

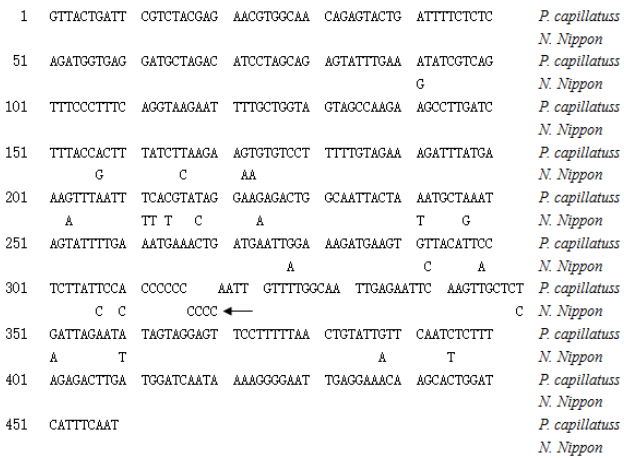

Fig. 2. Comparison of *N. nippon* partial CHD-W sequences with sequences of *P. capillatuss*. The "CCCC" sequence (where the arrow is pointing) was present in the CHD-W fragment of *N. nippon* but absent in *P. capillatuss's*

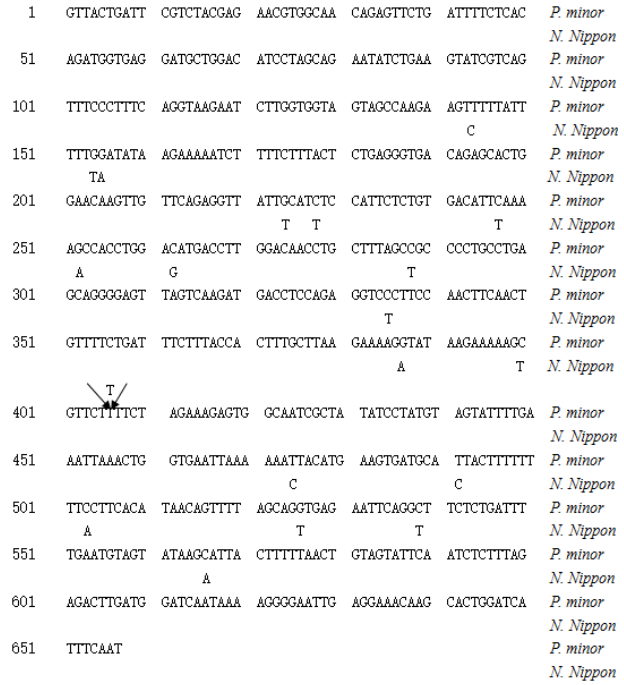

Fig. 3. Comparison of *N. nippon* partial CHD-Z sequences with sequences of *P. minor*. There was a "T" base (where the arrow is pointing) in *N. nippon's* less than in *P. minor's*.

In order to facilitate biodiversity and save *N. nippon* from extinction, captive breeding has been employed by Chinese government (Wang *et al*., 2010). During the period of the artificial breeding, we need to have the birds matched, which demand that we must know each individual sex in advance. It is usually difficult to distinguish female and male by general appearance of *N. nippon*. To solve this problem effectively, a reliable and effective method of sex identification is necessary. It is well known that the system determined the sex of birds is the ZW sex-determination system, where female birds have a ZW (as opposed to ZZ) sex chromosome. CHD-W gene links to W chromosome and its related gene CHD-Z links to Z chromosome (Halverson and Dvorak, 1993). There are some differences in structure and size between CHD-W and CHD-Z gene. Therefore, we can use the universal primers 2550F and 2718R for non-ratite bird sex identification to establish a molecular method for sex identification of *N. nippon* on the basis of the system optimization. There were several amplified fragments generated whether male or female, which might be related to the specificity of primers and didn't affect the results. The significant difference in agarose gel electrophoresis between the female and male samples enables this method to be used for correct sex determination of *N. nippon*.

Conclusion

Over 50% of all the world's bird species including *N. nippon* are monomorphic. It is very difficult to distinguish sex of those birds due to lack of sexual dimorphism (Vučićević *et al*., 2012). Most of the traditional methods of sex identification are not efficient. Molecular techniques, particularly those based on PCR, are feasible for sex identification in birds.

The objective of the present study is to establish the molecular methods of sex identification of *N. nippon* and provide technical support for the monitoring and protection of endangered birds. For this purpose some molecular operations such as cloning, sequencing and comparison to the sex-related genes were performed. The results indicated that the sexing universal primers 2550F/2718R had the ability to be used to check and identify the gender of *N. nippon*. Two sexing related genes fragments, a 657 bp CHD fragments on Z chromosome in both sexes and a 463 bp female specific CHD fragments on W chromosome were similar with CHD-W of *P. capillatuss* and CHD-Z of *P. minor* individually. This study is helpful for the management and conservation of the endangered *N. nippon*.

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Author's Contributions

Feilong Sun: Performed the experiments and wrote the manuscript.

Xuanfu Huang: Performed the experiments.

Shuiyun Yang: Developed the idea and analyzed the data.

Ethics

The authors declare that they have no conflict of interest. All authors have read and approved the manuscript and no ethical issues involved.

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